

# Free Radicals, Oxidative Stress, and Antioxidants in Human Health and Disease

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**ABSTRACT:** Free radicals and other reactive oxygen species (ROS) are constantly formed in the human body. Free-radical mechanisms have been implicated in the pathology of several human diseases, including cancer, atherosclerosis, malaria, and rheumatoid arthritis and neurodegenerative diseases. For example, the superoxide radical ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) are known to be generated in the brain and nervous system *in vivo*, and several areas of the human brain are rich in iron, which appears to be easily mobilizable in a form that can stimulate free-radical reactions. Antioxidant defenses to remove  $O_2^-$  and  $H_2O_2$  exist. Superoxide dismutases (SOD) remove  $O_2^-$  by greatly accelerating its conversion to  $H_2O_2$ . Catalases in peroxisomes convert  $H_2O_2$  into water and  $O_2$  and help to dispose of  $H_2O_2$  generated by the action of the oxidase enzymes that are located in these organelles. Other important  $H_2O_2$ -removing enzymes in human cells are the glutathione peroxidases. When produced in excess, ROS can cause tissue injury. However, tissue injury can itself cause ROS generation (e.g., by causing activation of phagocytes or releasing transition metal ions from damaged cells), which may (or may not, depending on the situation) contribute to a worsening of the injury. Assessment of oxidative damage to biomolecules by means of emerging technologies based on products of oxidative damage to DNA (e.g., 8-hydroxydeoxyguanosine), lipids (e.g., isoprostanes), and proteins (altered amino acids) would not only advance our understanding of the underlying mechanisms but also facilitate supplementation and intervention studies designed and conducted to test antioxidant efficacy in human health and disease.

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**KEY WORDS:** Antioxidants, atherosclerosis, DNA damage, flavonoids, free radicals, 8-hydroxydeoxyguanosine, isoprostanes, lipid peroxidation, oxidative protein damage, oxidative stress, phytochemicals.

Humans and other aerobes are able to tolerate oxygen ( $O_2$ ) because, at the same time that organisms were evolving electron transport chains and other enzyme systems to use it, antioxidant defenses to protect against the toxic effects of  $O_2$  were evolving in parallel. The predecessors of the anaerobic bacteria that exist today followed the “blind” evolutionary path of restricting themselves to environments that  $O_2$  did not

penetrate. Hence, the evolution of multicellular aerobes and of antioxidant defense mechanisms is intimately related.

Interest in free radicals began with the work of Moses Gomberg (1), who in 1900 demonstrated the existence of the triphenylmethyl radical ( $Ph_3C\cdot$ ). A free radical is any chemical species (capable of independent existence) that possesses one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. Radicals are generally less stable than nonradicals, although their reactivities vary. The simplest free radical is an atom of the element hydrogen. The hydrogen atom contains one proton and a single unpaired electron, which qualifies it as a free radical—often denoted by the insertion of the radical dot ( $\cdot$ ) to indicate that one or more unpaired electrons is present.

Once radicals form, they can react either with another radical or with another molecule by various interactions. The rate and selectivity of these types of reactions depend on high concentrations of the radicals, on delocalization of the single electron of the radical (thus increasing its life time), and on the absence of weak bonds in any other molecules present with which the radical could interact (see Table 1). Most biological molecules are nonradicals that contain only paired electrons. Extensive discussions on free-radical chemistry may be found elsewhere (2–7).

Gerschman *et al.* (8) proposed that “oxygen poisoning and radiation injury have at least one common basis of action, possibly the formation of oxidizing free radicals.” This pioneering idea soon began to capture the imaginations of scientists. In the early 1960s, superoxide was found to be associated with a number of enzymes, including xanthine oxidase. However, it was believed that this free radical was “bound” to the enzyme. In 1968, it was discovered that superoxide was secreted into solution (9–11), allowing superoxide to mediate cellular toxicity.

The role of free-radical reactions in human disease, biology, toxicology, and the deterioration of food has become an area of intense interest. In the main, the free-radical reaction of lipid peroxidation is an important issue in the food industry where manufacturers minimize oxidation in lipid-containing foods by use of antioxidants during the manufacturing process; foods are produced that maintain their nutritional quality over a defined shelf life. By contrast, biomedical sci-

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**TABLE 1**  
**Chemical Reactions of Free Radicals<sup>a</sup>**

### Unimolecular Radical Reactions

Reactions result from the instability of the first formed radical. The radicals may completely decompose or rearrange before reaction with other molecules or radicals present.

Decomposition:

Reaction in which the radical decomposes to give a stable molecule and a new radical.

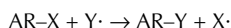
Rearrangement:

1. Breaking of an adjacent C–C bond in a cyclic system with concomitant formation of a new bond, usually carbonyl and a new isomeric radical.
2. Migration of an atom, *via* intramolecular abstraction by the radical center, thus creating a new, isomeric radical.

### Radical-Molecule Interactions

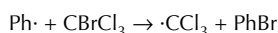
*Addition to unsaturated systems:*

1. Addition of a radical to an olefinic double bond to give a new, saturated radical. Typical reaction is the radical-induced polymerization of olefins.
2. Addition of a radical to an aromatic double bond. This intermediate step is widespread in free-radical chemistry, e.g., in the radical substitution of aromatic compounds (homolytic aromatic substitution). The net overall reaction is displacement of an aromatic substituent by a radical:



*Abstraction or displacement: S<sub>H</sub>2 reactions<sup>b</sup>:*

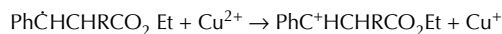
1. Biomolecular reaction involving homolytic attack of a radical on a molecule. The radical attacks a univalent atom, usually a terminal halogen or hydrogen in an abstraction reaction to give rise to a new radical, e.g.,



2. Homolytic substitution at multivalent atoms also occurs, but both do not normally occur at saturated carbon centers.

*Reaction with oxidizing agents:*

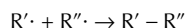
Radicals readily undergo 1-electron oxidations with oxidizing reagents of suitable redox potential to give positive ions. Example is the Meerwein reaction, which involves the oxidation of cinnamyl-derived radicals by cupric ions:



### Radical-Radical Interactions

*Dimerization or radical coupling:*

Localized radicals (methyl, phenyl radicals) react readily with little chance of dimerization. Only delocalized radicals have a high probability of dimerization in solution. Thus,



When  $\text{R}' = \text{R}''$ , the reaction is dimerization; and when  $\text{R}' \neq \text{R}''$ , the reaction is radical coupling or combination.

*Radical disproportionation:*

Involves collision of the radicals, resulting in the abstraction of an atom, usually hydrogen, by one radical from the other. This leads to the formation of two stable molecules, with the atom abstracted being  $\beta$  to the radical center:<sup>c</sup> e.g., the disproportionation of two phenylethyl radicals to give styrene and ethylbenzene.

<sup>a</sup>The reader is referred to References 2–7 for an extensive overview of the reaction sequences highlighted in this table and an examination of the complex chemistry of organic radicals.

<sup>b</sup>S<sub>H</sub>2 stands for substitution homolytic biomolecular.

<sup>c</sup>The disproportionation reaction derives its driving force from the formation of two new strong bonds and from the fact that the  $\beta$ -CH bonds in radicals are usually weak.

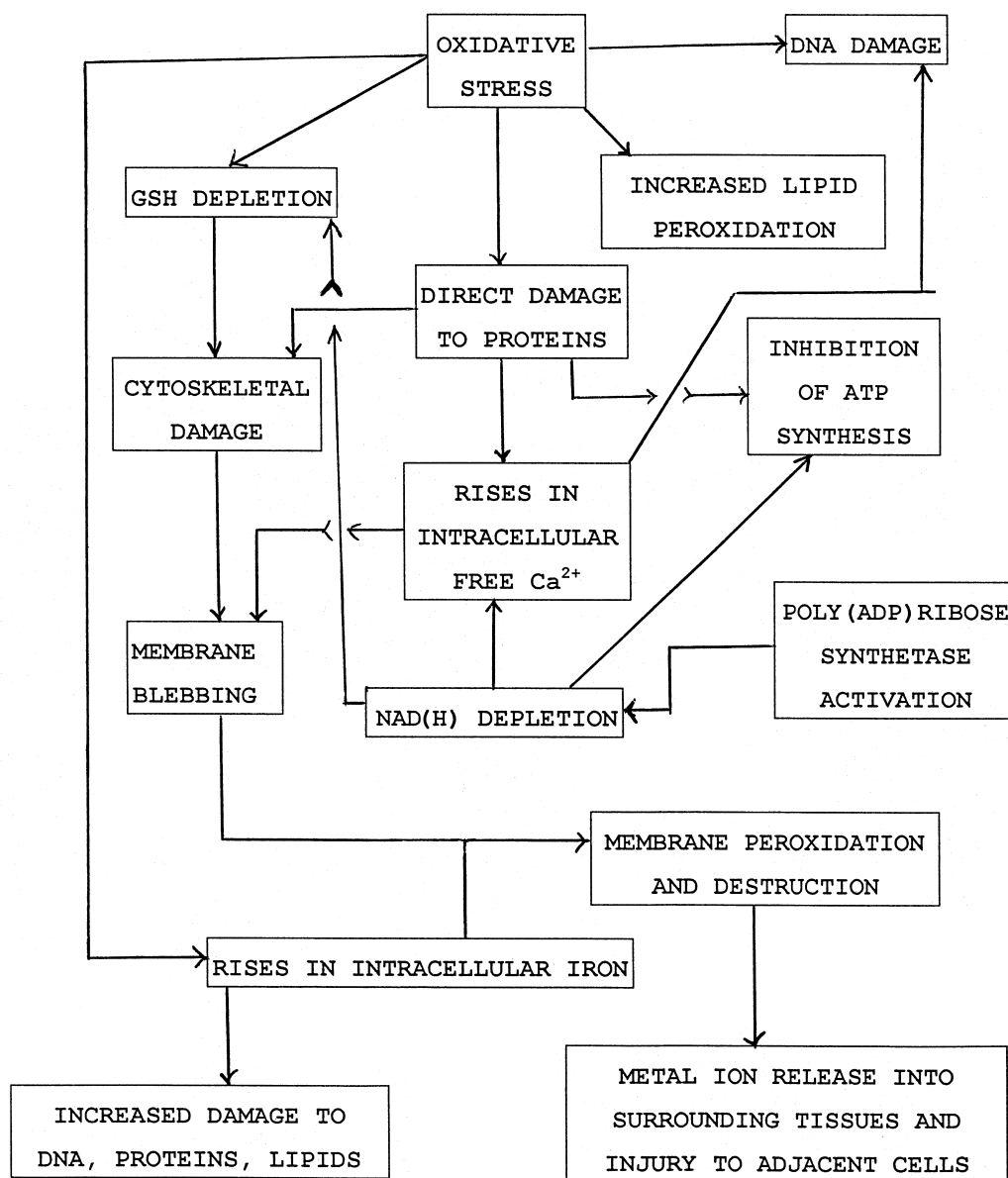
entists and clinicians are interested in antioxidants because they protect the body against damage by reactive oxygen species (11–21). The rest of this article will focus on biomedical aspects of free radicals.

## REACTIVE OXYGEN SPECIES

Free radicals of importance in living organisms include hydroxyl (OH·), superoxide (O<sub>2</sub><sup>·-</sup>), nitric oxide (NO·), and peroxy (RO<sub>2</sub>·). Peroxynitrite (ONOO<sup>-</sup>), hypochlorous acid

(HOCl), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), singlet oxygen (<sup>1</sup>Δg (often written as <sup>1</sup>O<sub>2</sub>), and ozone (O<sub>3</sub>) are not free radicals but can easily lead to free-radical reactions in living organisms. The term “reactive oxygen species” (ROS) is often used to include not only the radicals OH·, RO<sub>2</sub>·, NO· and O<sub>2</sub><sup>·-</sup> but also the nonradicals HOCl, <sup>1</sup>O<sub>2</sub>, ONOO<sup>-</sup>, O<sub>3</sub>, and H<sub>2</sub>O<sub>2</sub>.

Extensive reviews on the role of free radicals in biology and medicine abound (22–30). Scheme 1 summarizes the mechanisms of cellular damage in oxidative stress. “Oxidative stress” is the term that refers to the imbalance between



SCHEME 1

generation of ROS and the activity of the antioxidant defenses. Severe oxidative stress can cause cell damage and death. It has been implicated in numerous human diseases (Table 2). In mammalian cells, oxidative stress appears to lead to increases in the levels of free  $\text{Ca}^{2+}$  and iron within cells (31,32). Indeed, Orrenius *et al.* (31) have suggested that excessive rises in intracellular free  $\text{Ca}^{2+}$  may cause DNA fragmentation by activating endonucleases. The importance of oxidative stress injury depends on the molecular target, the severity of the stress, and the mechanism by which the oxidative stress is imposed, i.e., drug-induced, Fenton chemistry, trauma, enzyme activation [e.g.,  $\text{NO}\cdot$  synthase (NOS) activity], etc. Brief comments on some of the ROS are worthwhile.

**Ozone.** This pale blue gas, which is not produced *in vivo*, serves as an important protective shield against solar radiation in the atmosphere. Close to the earth's surface,  $\text{O}_3$  is an

unwanted oxidant and is often regarded as the most toxic air pollutant (33).  $\text{O}_3$  can form in laboratory equipment that has high-energy ultraviolet (UV)-producing lamps and in urban air as a result of photochemical reactions and pollution. The tissue most susceptible to damage upon exposure to  $\text{O}_3$  is the lung. The biological effect of  $\text{O}_3$  is often attributed to its ability to cause oxidation or peroxidation of biomolecules either directly or *via* free-radical mechanisms (34–36).

**Nitric oxide (NO).**  $\text{NO}\cdot$  is a free radical. It is widely thought that the endothelium-derived relaxing factor (EDRF) produced by vascular endothelium, which is an important mediator of vascular responses induced by several pharmacological agents (including bradykinin and acetylcholine), is identical to  $\text{NO}\cdot$  (37,39). Vascular endothelial cells that line the blood vessels also seem to produce small amounts of  $\text{O}_2^{\cdot-}$ , some of which could react with NO to produce nonradical

**TABLE 2**  
**Some Clinical Conditions in Which Free Radicals and Other Reactive Oxygen Species**  
**Are Thought to Be Involved<sup>a,b</sup>**

BRAIN	INFLAMMATORY IMMUNE INJURY
Parkinson's disease	Rheumatoid arthritis
Neurotoxins	Glomerulonephritis
Vitamin E deficiency	Autoimmune diseases (HIV/AIDS)
Hyperbaric oxygen	Vasculitis (hepatitis B virus)
Hypertensive cerebrovascular injury	ALCOHOLISM
Aluminum overload	AGING
Allergic encephalomyelitis (demyelinating diseases)	Age-related macular degeneration
Potential of traumatic injury	RADIATION INJURY
EYE	IRON OVERLOAD
Photoc retinopathy	Nutritional deficiencies (kwashiorkor)
Ocular hemorrhage	Thalassemia and other chronic anemias treated with multiple blood transfusions
Cataractogenesis	Dietary iron overload (red wine, beer brewed in iron pots)
Degenerative retinal damage	Idiopathic hemochromatosis
Retinopathy of prematurity	RED BLOOD CELLS
HEART AND CARDIOVASCULAR SYSTEM	Fanconi's anemia
Atherosclerosis	Sickle cell anemia
Adriamycin cardiotoxicity	Favism
Keshan disease (selenium deficiency)	Malaria
Alcohol cardiomyopathy	Protoporphyrin photo-oxidation
KIDNEY	LUNG
Metal ion-mediated nephrotoxicity	Bronchopulmonary dysplasia
Aminoglycoside nephrotoxicity	Mineral dust pneumoconiosis
Autoimmune nephrotic syndromes	Bleomycin toxicity
GASTROINTESTINAL TRACT	Hypoxia
NSAID-induced GI tract lesions <sup>a</sup>	Cigarette smoke effect
Oral iron poisoning	Emphysema
Endotoxin liver injury	ARDS (some forms)
Diabetogenic actions of alloxan	Oxidant pollutants (O <sub>3</sub> , SO <sub>2</sub> , NO <sub>2</sub> )
Halogenated hydrocarbon liver injury	ISCHEMIA-REPERFUSION
FFA-induced pancreatitis	Stroke/myocardial infarction
CANCER	Organ transplantation
	AMYOTROPHIC LATERAL SCLEROSIS

<sup>a</sup>Abbreviations: ARDS, adult respiratory distress syndrome; NSAID, nonsteroidal antiinflammatory drug; FFA, free fatty acid.

<sup>b</sup>Skin injury due to solar radiation, porphyria, contact dermatitis, and photosensitizers may also involve free-radical mechanisms.

products. Thus, variation in the production of NO· and O<sub>2</sub><sup>-</sup> by endothelium might provide one mechanism for the regulation of vascular tone and hence blood pressure.

A role for NO· has also been demonstrated in such human diseases as malaria, where NO· appears to be partly involved in resistance to malaria infection, in cardiovascular disease, acute inflammation, cancer, neurodegenerative diseases, and diabetes. NO· exerts potent actions in the regulation of cell function and tissue viability—this includes the recognized ability to mediate signal transduction *via* stimulation of guanylate cyclase-mediated cGMP synthesis (reviewed in Refs. 37–43). Sessa *et al.* (44) advocated that in dogs, “chronic exercise, presumably by increasing endothelial shear stress, increases EDRF/NO· production and endothelial cell NO· synthase gene expression, and may contribute to the beneficial effects of exercise on the cardiovascular effort.” The reaction between NO· and O<sub>2</sub><sup>-</sup> leads to DNA oxidative damage owing to the formation of ONOO<sup>-</sup>, which may have OH-like potential, leading to the formation of nitroguanine and other loose products (45–51). ONOO<sup>-</sup> has the propensity to

damage supercoiled DNA in plasmid pBR322 (52). A suggested toxicity of NO· involves its reaction with O<sub>2</sub><sup>-</sup> to give ONOO<sup>-</sup>, a reaction that occurs with a rate constant of 6.7 × 10<sup>9</sup> M<sup>-1</sup>s<sup>-1</sup> (53). ONOO<sup>-</sup> has been suggested to mediate NO-dependent toxicity. In addition to DNA base nitration mentioned above, ONOO<sup>-</sup> potentiates the activation of guanylate cyclase; has bactericidal activity and trypanocidal activity, converting low-density lipoprotein (LDL) to a form that may be recognized by the macrophage scavenger receptor; induces peroxidation of lipids; oxidizes methionine and SH residues in proteins; depletes antioxidants (e.g., ascorbate, glutathione), nitrates tyrosine residues; and inactivates α<sub>1</sub>-antiproteinase (α<sub>1</sub>AP) (the major inhibitor of serine proteases in human body fluids). Indeed, nitration of tyrosine residues appears to be a “marker” of ONOO<sup>-</sup>-dependent damage *in vivo* (42,54–63).

*Peroxy radicals (RO<sub>2</sub>·)*. These radicals are intermediate species formed during lipid oxidation chain reactions, such as the oxidation of polyunsaturated fats, resulting in deterioration of lipid-containing foods. Lipid peroxidation may be ini-

tiated by any species that has sufficient reactivity to abstract a hydrogen atom from a polyunsaturated fatty acid (PUFA) side chain (such as those of arachidonic acid and linolenic acid, for example) in membrane lipids. Arachidonic acid is a precursor of prostaglandins and leukotrienes. It contains a number of methylene-interrupted double bonds, which are particularly prone to hydrogen atom abstraction (64). Although much has been written about the mechanism and biological significance of lipid peroxidation, there seems to be no unanimity in the methods for its measurement. Delineation of lipid peroxidation as a major pathway in most degenerative diseases (Table 2) would depend on adequate standardization and control of measurement conditions. The link between DNA damage, faulty repair of DNA, proto-oncogene activation, and the ability of some of the end-products of lipid peroxidation to act as promoters of carcinogenesis is widely discussed (65,66).

End-products of lipid peroxidation could have profound effects on vascular function because of their ability to mimic or antagonize the actions of some of the stereospecific products formed by cyclooxygenase and lipoxygenase enzymes. For example, the  $F_2$ -isoprostanes are generated by the peroxidation of arachidonic acid *via* the generation of  $RO_2\cdot$  isomers, which undergo endocyclization to prostaglandin-like compounds. Their formation *in vivo* appears to be enhanced under conditions of oxidative stress, such as smoking, exposure to xenobiotics, and pathological conditions associated with inflammation (67,68). The mechanism of LDL oxidation possesses the general characteristics of lipid peroxidation reactions and free-radical reactions (69).

*Hypochlorous acid.* HOCl is produced by the neutrophil-derived enzyme myeloperoxidase at sites of inflammation and when activated neutrophils infiltrate reoxygenated tissue (28). The enzyme oxidizes chloride ions,  $Cl^-$ , in the presence of  $H_2O_2$ . A total of  $5 \times 10^6$  activated human neutrophils in 1 mL produces 88  $\mu M$  HOCl in 2 h at 22°C (70). HOCl is not a free radical, but it is a potent chlorinating and oxidizing agent. It has recently been suggested that the formation of cholesterol chlorohydrins could disrupt cell membranes and lead to cell lysis and death (71). On the basis of this observation, the cholesterol chlorohydrins have been suggested to be potential biomarkers for oxidative damage associated with neutrophil/monocyte activation (71). HOCl can attack many other biological molecules. For example, the proteolytic inhibitor alpha-1-antitrypsin ( $\alpha_1AP$ ) is the major inhibitor in human plasma of proteolytic enzymes, such as elastase.  $\alpha_1AP$  accounts for about 90% of the elastase-inhibitory capacity of the human serum (72). Thus, its inactivation by HOCl might greatly potentiate tissue damage because elastase is also released from activated neutrophils. HOCl attacks primary amines and sulfhydryl groups in proteins and chlorinates purine bases in DNA (73,74). Kozumbo *et al.* (75) have demonstrated that physiological levels of HOCl can react with substituted aryl amine (e.g., aniline, 1-naphthylamine, and 1-naphthol) to form long-lived products that bind DNA and are genotoxic to human cells.

In the context of food handling, HOCl is a major active constituent of chlorine-based bleaches that are often used to disinfect equipment with which foods will come into contact. Perhaps most significant is the observation that phenolic compounds can react effectively with HOCl, hence protecting susceptible targets against oxidation (76,77). This may have physiological significance, given the interest in the use of dietary natural antioxidants to manipulate disease states (78).

*Superoxide radicals ( $O_2^{\cdot-}$ ).*  $O_2^{\cdot-}$  is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems, by autooxidation reactions, and by nonenzymatic electron transfers that univalently reduce molecular oxygen. In aqueous solution,  $O_2^{\cdot-}$  can oxidize ascorbic acid. It can also reduce certain iron complexes such as cytochrome c and ferric-ethylenediaminetetraacetic acid ( $Fe^{3+}$ -EDTA). Superoxide dismutase (SOD) accelerates the dismutation of  $O_2^{\cdot-}$ , converting it to  $H_2O_2$  and  $O_2$ . For an authoritative review, see References 10 and 79.

*Hydrogen peroxide ( $H_2O_2$ ).* Like HOCl and  $O_3$ ,  $H_2O_2$  is not a free radical. It can cross membranes and may slowly oxidize a number of compounds.  $H_2O_2$  can be formed *in vivo* when  $O_2^{\cdot-}$  dismutates and also by many oxidase enzymes.  $H_2O_2$  at low (micromolar) levels also seems to be poorly reactive. However, higher levels of  $H_2O_2$  can attack several cellular energy-producing systems; for example, it inactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase.  $H_2O_2$  also forms  $OH\cdot$  in the presence of transition metal ions, and  $O_2$  can facilitate this reaction.

*Hydroxyl radicals.*  $OH\cdot$  is a highly reactive oxygen-centered radical with an estimated half-life in cells of only  $10^{-9}$  s. Most studies on  $OH\cdot$ , prior to the discovery of SOD, were done by radiation chemists. One feature of  $OH\cdot$  is that it begets another radical, i.e., when it reacts with a molecule, the result is the formation of another radical species. The resulting species usually has lower reactivity than  $OH\cdot$ .  $OH\cdot$  attacks all proteins, DNA, PUFA in membranes, and almost any biological molecule it touches.

In the case of  $OH\cdot$  generation by Fenton-type chemistry, the extent of  $OH\cdot$  formation is largely determined by the availability and location of the metal ion catalyst. In an *in vitro* study, in which the technique of gas chromatography-mass spectrometry with selected ion monitoring (GC/MS/SIM) was used to compare the role of copper and iron ions in promoting damage to DNA by  $H_2O_2$ , added copper ions (80) were significantly more reactive in causing DNA damage in the presence of  $H_2O_2$  than were equimolar iron ions. The availability of "free" iron and copper *in vivo* is controlled. Iron ions are absorbed from the gut and transported to iron-requiring cells by the protein transferrin. Iron specifically bound to transferrin does not participate in free-radical reactions. Excess iron is stored as ferritin and hemosiderin in an attempt to keep the iron pool as small as possible. Most or all of the plasma copper in humans is attached to the protein ceruloplasmin, which will not stimulate free-radical reactions under normal conditions (81,82). Hydroxyl radical generation can take place when the hemostasis is altered.

For example, copper and iron ions released into perfusates can cause ischemia/reperfusion injury (83). Ramos *et al.* (84), using the electron spin resonance (ESR) spin-trapping system, detected OH· as the  $\alpha$ -hydroxyethyl spin-trapped adduct of 4-pyridyl 1-oxide *N-tert*-butylnitron, formed from phorbol 12-myristate 13-acetate-stimulated human neutrophils and monocytes without the addition of supplemental iron. In systems that lacked myeloperoxidase, it is necessary to add iron to detect the OH· adduct. Thus, human neutrophils and monocytes can generate OH· through a myeloperoxidase-dependent mechanism (85), which could have microbicidal implications.

Tissue injury can itself cause ROS generation (e.g., by causing activation of phagocytes or releasing transition metal ions from damaged cells), which may (or may not, depending on the situation) contribute to a worsening of the injury. For example, the sequelae of traumatic brain injury and stroke may involve a postinjury stimulation of iron ion-dependent free-radical reactions. Parkinson's disease is caused by death of cells in the substantia nigra. Lysis of dead cells could cause iron ion release. Thus, patients with Parkinson's disease may be under oxidative stress, and free-radical reactions probably contribute to the degeneration of the substantia nigra (86).

### PROTECTION AGAINST ROS-INDUCED DAMAGE

Some generation of ROS *in vivo* is unavoidable and may be deliberate; NO· is a typical example. The most important route for the formation of NO· is the oxidation of arginine by NOS of which there are two types: the constitutive calcium/calmodulin-dependent forms (cNOS), which produce low levels of NO·, and the inducible forms (iNOS). The iNOS, which have tightly bound calmodulin, are permanently active and are capable of generating high levels of NO· (37,38,42,43,87). The phagocytes (neutrophils, monocytes, macrophages, eosinophils) that defend against foreign organ-

isms are also involved in this good and bad scenario. They generate O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and (in the case of neutrophils) HOCl as one of their mechanisms for killing foreign organisms (26,27). This essential defense mechanism can go awry: several diseases (such as rheumatoid arthritis and inflammatory bowel disease) are accompanied by excessive phagocyte activation and resulting tissue damage, to which ROS contribute.

Human defenses against ROS-induced damage include the enzymes catalase and glutathione peroxidase (both of which remove H<sub>2</sub>O<sub>2</sub>, as well as SOD, which catalyzes the dismutation of O<sub>2</sub><sup>-</sup> to form H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>). Glutathione peroxidase is generally thought to be more important than catalase as a H<sub>2</sub>O<sub>2</sub>-removing system in humans. Catalase is located in peroxisomes, whereas glutathione peroxidase is localized in the mitochondria and cytosol, a similar distribution to that of SOD. Interestingly, in mammalian (including human) tissues, SOD is mainly an intracellular enzyme. Only small amounts are present in extracellular fluids, such as plasma, cerebrospinal fluid or synovial fluid. In addition, some low-molecular-mass substances, such as uric acid, ascorbate (vitamin C), glutathione, tocopherol (vitamin E), ubiquinol, ergothioneine, hypotaurine, and lipoic acid, may act as antioxidants in the human body. Much has been written about the antioxidant status of the biological system. References 17, 19, 78, and 88–98 are illustrative.

The rates of free-radical generation probably increase in most diseases, although we still do not know how important they are in promoting tissue injury, mainly because of the limitations of the assays used to measure them. The development and validation of assays, indicated in Table 3 for humans, should allow us to rapidly evaluate the role of free radicals in disease pathology and establish a logical basis for the therapeutic use of antioxidants. Numerous antioxidant supplementation studies (17,18,99–117) for the primary prevention of chronic diseases have been undertaken. The principal endpoint has always been "incidence" of the respective disease,

**TABLE 3**  
**Measurement of Oxidative Damage in Humans<sup>a</sup>**

OXIDATIVE DNA DAMAGE
Gas chromatography–mass spectrometry–selected ion monitoring detection of oxidized base products
High-performance liquid chromatography-based assays for oxidized base products
Single gel electrophoresis assay (COMET ASSAY)
OXIDATIVE DAMAGE TO LIPIDS
Measurement of conjugated dienes
Measurement of isoprostanes
Measurement of hydroperoxides
Measurement of thiobarbituric acid-reactive materials by HPLC
ASSESSMENT OF THE LEVELS OF ANTIOXIDANT ENZYMES
Catalase, superoxide dismutase, and glutathione peroxidase
ASSESSMENT OF PROTEIN DAMAGE
Steady-state protein damage can be quantified in terms of the numbers of protein carbonyls and modified tyrosine residues. Total ongoing (repaired) protein damage can be indicated by the concentration of modified tyrosines and fluorescent bityrosines in urine.
ASSESSMENT OF LEVELS OF LOW MOLECULAR WEIGHT ANTIOXIDANTS AND VITAMINS
Uric acid/allantoin, glutathione, flavonoids, vitamins E and C, $\beta$ -carotene

<sup>a</sup>There are several indicators of the extent of oxidative damage in humans. Only the most common are included in this table.

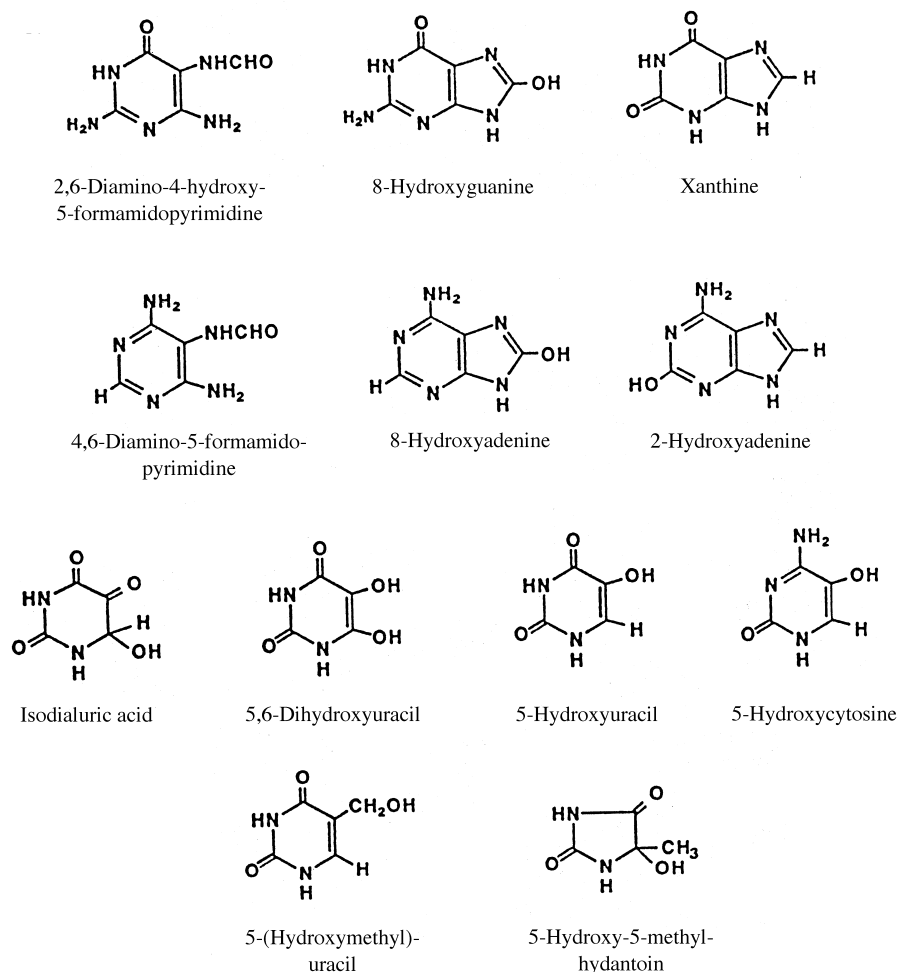
i.e., incidence of cancer or cardiovascular disease. In a departure from this, Duthie *et al.* (20) investigated the extent of oxidative DNA damage in Scottish men aged 50–59 years. Their result suggests that long-term antioxidant supplementation can decrease both endogenous and exogenous oxidative DNA damage in lymphocytes. I suggest that the application and validation of the biochemical markers in Table 3 should be built into future supplementation studies to evaluate the pharmacology of antioxidants (drug- or plant-derived antioxidants). It is necessary to balance this with the choice of population, formulation and dose of antioxidants being used, the expected outcome variables, and the pathologic end points.

### MEASURING OXIDATIVE DAMAGE *IN VIVO*

The remainder of this article will focus on the technology (emerging and established) for assessing the products of oxidative damage to DNA, proteins, and lipids in context human diseases.

*Measurement of oxidative DNA damage.* Oxidative damage to DNA seems to occur continuously *in vivo*, in that low levels (presumably a “steady-state” balance between DNA

damage and repair) have been detected in DNA isolated from human cells and tissues. The pattern of damage to the purine and pyrimidine bases bears the chemical fingerprint of OH $\cdot$  attack, suggesting that OH $\cdot$  formation occurs within the nucleus *in vivo* (117–123) (Scheme 2, DNA base products). However, this raises the question of how OH $\cdot$  could be produced in the nucleus. If OH $\cdot$  is attacking DNA, it must be produced close to the DNA because OH $\cdot$  is so reactive that it cannot diffuse from its site of formation. Background radiation may be one source, but radiation-generated OH $\cdot$  is formed over the whole cell and only a small fraction hits DNA (119). Other potential sources of OH $\cdot$  or OH $\cdot$ -like species include the decomposition of ONOO $^-$ , the reaction of O $_2^-$  with HOCl, the attack of HOCl on DNA bases, and generation of chlorinated products. The greatest interest has been in reactions of transition-metal ions with H $_2$ O $_2$  as a source of OH $\cdot$  (80, 120–122). The key question is whether “catalytic” transition-metal ions (e.g., iron and copper ions) are really in close proximity to DNA *in vivo*. Although iron and copper seem to be present in the nucleus (123,124), it remains to be established why and how they reach the DNA because these ions are normally carefully sequestered by the human body.



SCHEME 2

However, oxidative stress and cell death can liberate metal ions from their normal sequestration sites and might then bind to DNA, a powerful metal ion chelator. Extensive reviews on the chemistry and measurement of oxidative DNA damage may be found in References 120 and 125. Base products may be measured essentially as described in References 126–130.

There are two types of measurement of oxidative DNA damage. Steady-state damage can be measured when DNA is isolated from human cells and tissues and analyzed for base damage products: it presumably reflects the balance between damage and DNA repair. Hence, a rise in steady-state oxidative DNA damage could be due to increased damage and/or decreased repair. The measurement of baseline levels of oxidatively modified DNA bases, although important, does not necessarily provide information as to whether this damage is in active genes or quiescent DNA. It is important also to have an index of total DNA damage in the human body, i.e., the “input” side of the steady-state equation. The most common approach has been to assess the “output” side, i.e., trying to estimate the rate of repair of oxidized DNA.

Several DNA base damage products are excreted in human urine, including the nucleoside 8-hydroxy-deoxyguanosine (8-OHdG), 8-hydroxy-adenine, and 7-methyl-8-hydroxyguanine (131–133), but the one most exploited is 8-OHdG, usually measured by a method involving high-performance liquid chromatography (HPLC) with electrochemical detection. GC/MS has also been used to measure 8-OHdG in urine, and the limit of detection was 1.8 pmol, corresponding to a level of 8-OHdG in urine of 35 nM (134). The level of 8-OHdG in urine is presumably unaffected by the diet because nucleosides are not absorbed from the gut. The question of whether any 8-OHdG is metabolized to other products in humans has not been rigorously addressed. It is also possible that some or all of the 8-OHdG excreted in urine may arise not from DNA, but from deoxyGTP (dGTP) in the DNA precursor pool of nucleotides. An enzyme has been described that hydrolyzes dGTP-containing oxidized guanine to prevent its incorporation into DNA (135,136). The development of alternative urinary markers of total-body oxidative damage, to address these uncertainties and to validate measurements of guanine damage products in DNA by HPLC and GC/MS is the focus of current investigations (reviewed in Ref. 137).

## LIPIDS: LIPID PEROXIDATION AND THEIR MEASUREMENT

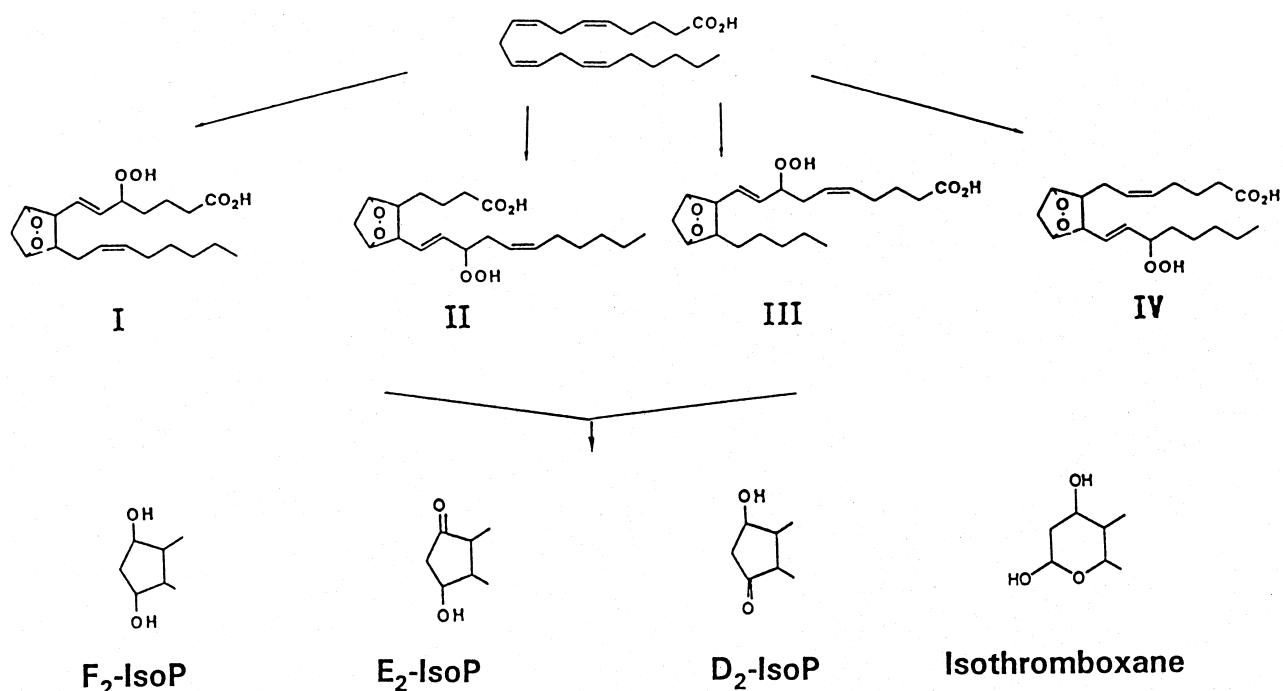
Lipid peroxidation is important *in vivo*. It contributes to the development of cardiovascular diseases, such as preeclampsia and atherosclerosis, and the end-products of this process [particularly cytotoxic aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE)] can cause damage to proteins and to DNA. Peroxidation causes impairment of biological membrane functioning, e.g., decreases fluidity, inactivates membrane-bound enzymes and receptors, and may change nonspecific calcium ion permeability (31,32). The more unsaturated a fatty acid side-chain, the greater its

propensity to undergo lipid peroxidation. But do PUFA really peroxidize faster than saturated fatty acids *in vivo*? This question is particularly important in relation to proposals that increases in dietary polyunsaturate/saturate ratios are beneficial to health (reviews in 64,66,137). The ability to measure levels of isoprostanes and hydroxyeicosatetraenoic acids (HETE) represents an important development in attempts to measure clinically relevant oxidative lipid damage. The hydroperoxides HETE and isoprostanes are biologically active. HETE are chemotactic for neutrophils and facilitate calcium uptake and protein kinase C mobilization (138, 139). The 12-HETE are involved in adrenocorticotropin and parathyroid secretion, modulation of mitogenic processes, and lymphocyte function (140–142), while the 15-HETE may inhibit neutrophil migration across cytokine-activated endothelium (143). HETE can be measured by GC–MS, operated in the negative ion chemical ionization mode (144–146). Isoprostanes (Scheme 3) are a series of prostaglandin-like compounds that are formed during peroxidation of arachidonic acid, although probably similar products are formed from other PUFA. Because they are structurally similar to prostaglandin  $F_{2\alpha}$ , the compounds are collectively referred to as  $F_2$ -isoprostanes. These compounds can be measured in plasma ( $35 \pm 6$  pg/mL) and urine ( $1600 \pm 600$  pg/mg creatinine) of healthy volunteers, indicative of ongoing lipid peroxidation even in healthy human subjects. Most of the plasma isoprostanes is esterified to phospholipids, but some are “free.” One of the isoprostanes, 8-*iso* PGF $_{2\alpha}$ , is a powerful renal vasoconstrictor and decreases kidney blood flow and glomerular filtration rate by almost half at low nanomolar concentrations. It has been suggested that elevated circulating concentrations of  $F_2$ -isoprostanes may contribute to the pathology of hepatorenal syndrome, an almost uniformly fatal disorder characterized by the development of kidney failure in patients with severe liver disease. Urinary excretion of isoprostanes is elevated in patients with scleroderma and in smokers (extensive reviews may be found in Refs. 67,68,144,147,148). Isoprostanes and their metabolites can be measured in human urine, and this may prove to be a valuable assay of whole-body lipid peroxidation if a confounding effect of diet can be ruled out. The mechanism of formation of the isoprostanes is shown in Scheme 3, including the pathways (without the stereochemical orientation for clarity) that lead to the formation of four regioisomers (I–IV) of isoprostanes. The endoperoxides can undergo reduction to produce  $F_2$ -isoprostanes or rearrangements to form the  $E_2$ -isoprostanes,  $D_2$ -isoprostanes, and/or isothromboxanes (68).

## OXIDATIVE DAMAGE TO PROTEINS AND THEIR MEASUREMENT

Oxidative damage to proteins *in vivo* may affect the function of receptors, enzymes, transport proteins, etc. and perhaps generate new antigens that provoke immune responses. Products of oxidative protein damage can contribute to secondary damage to other biomolecules, e.g., inactivation of DNA repair enzymes and loss of fidelity of DNA polymerases in





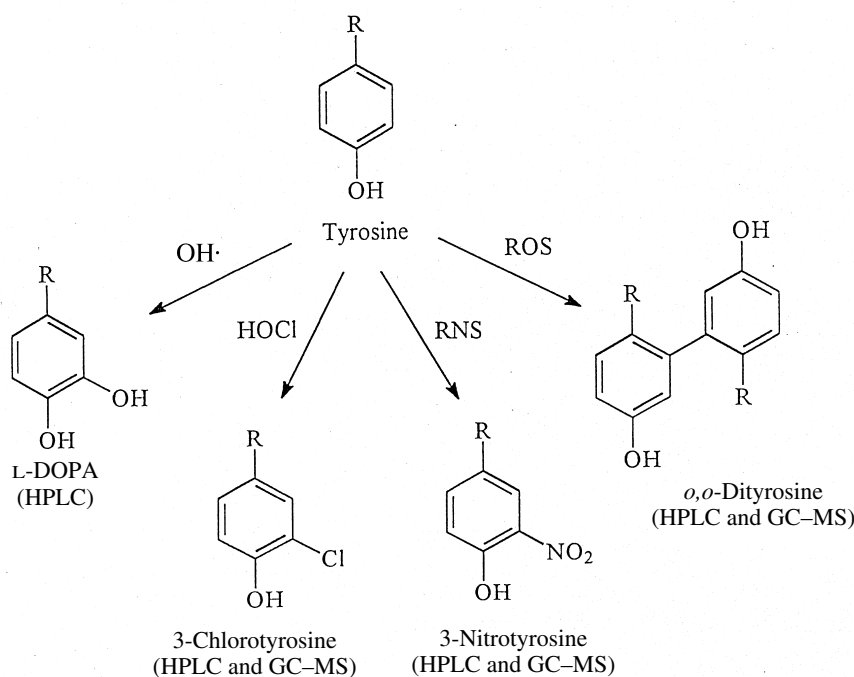
SCHEME 3. Reprinted by kind permission of Elsevier Science Inc. from Reference 68.

replicating DNA (149–151).

The chemical reactions that result from attack of ROS upon proteins are complex (152). Free-radical attack upon proteins generates radicals from amino acid residues, and electrons can be transferred between different amino acids (Scheme 4, products of oxidative protein damage) (137,149). The levels of any one (or, preferably, of more than one) of these products in proteins could in principle be used to assess

the balance between oxidative protein damage and the repair of (or, more likely, hydrolytic removal of) damaged proteins. The only product exploited to date has been hydroxylated phenylalanine. For example, levels of ortho-tyrosine and di-tyrosine in human lens proteins have been reported in relation to age (153). RNS in Scheme 4 refers to reactive nitrogen species, such as NO and peroxynitrite.

The carbonyl assay (154), a general assay of oxidative pro-



SCHEME 4

tein damage, to assess steady-state protein damage in human tissues and body fluids is based on the fact that several ROS can attack histidine, arginine, lysine, and proline residues in proteins to produce carbonyl functions that can be measured after reactions with 2,4-dinitrophenylhydrazine (DNPH) (149,155–158). Immunochemical methods involving anti-DNPH antibodies also have been described (159,160). Nevertheless, the reader is referred to the seminal discussions of the mechanisms and the molecular biology of oxidative damage to proteins (in Refs. 161–163).

ROS are formed in the human body. When endogenous antioxidant defenses are inadequate for the purpose of scavenging the ROS completely, ongoing oxidative damage to DNA, lipids, proteins and other molecules can be demonstrated. Although ROS have been implicated in pathology of several human diseases, it is important to appreciate that ROS may not be the primary cause of these diseases. Although diet-derived and drug-derived antioxidants may be particularly important in protecting against a number of human diseases (see Table 2), the physiological relevance of these effects is uncertain. The effects of dietary antioxidants and/or antioxidant supplements *in vivo* are best studied using a combination of validated methods (see Table 3) for measuring oxidative damage and where a particular disease is being investigated, the respective disease end point (164). This should now become part of the strategy for future antioxidant supplementation and intervention studies of any kind.

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